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(54) Title: BRASSICA PLANTS WITH HIGH LEVELS OF ANTICARCINOGENIC GLUCOSINOLATES

(57) Abstract: The present invention relates to a method for providing plants belonging to the Brassicaceae family with elevated levels of anticarcinogenic glucosinolates. The plants are obtained by 1) the production of a Brassica oleracea plant with elevated levels of anticarcinogenic glucosinolates in the edible parts and 2) the use of the Brassica oleracea plant produced under 1) as a starting material for breeding Brassica varieties with elevated levels of anticarcinogenic glucosinolates.

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**BRASSICA PLANTS WITH HIGH LEVELS OF ANTICARCINOGENIC
GLUCOSINOLATES**

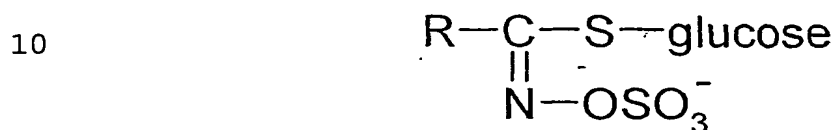
The present invention relates to a method for producing
5 plants belonging to the Brassicaceae family with elevated
levels of anticarcinogenic glucosinolates. The invention
further relates to Brassica plants that can be obtained using
the method according to the present invention as well as
their seeds and parts of plants. The invention further
10 relates to the use of Brassica plants for preparing food
products and/or pharmaceutical compositions that can be used
for prophylaxis and/or treatment of cancer.

The Brassicaceae family includes a large number of
important horticultural plants such as cauliflower or
15 romanesco (Brassica oleracea convar. botrytis var. botrytis);
broccoli (Brassica oleracea convar. botrytis var. cymosa);
broccoli sprout (Brassica oleracea convar. botrytis var.
asparagoides); Brussels sprouts (Brassica oleracea convar.
oleracea var. gemmifera); white cabbage or oxheart cabbage
20 (Brassica oleracea convar. capitata var. alba); red cabbage
(Brassica oleracea convar. capitata var. rubra); Savoy
cabbage (Brassica oleracea convar. capitata var. sabauda);
kohlrabi (Brassica oleracea convar. acephala var.
gongyloides); kale (Brassica oleracea convar. acephala var.
25 sabellica); and Portuguese cabbage (Brassica oleracea var.
trunchuda syn. costata).

The Brassicaceae family is characterised by the presence
of typical secondary metabolites that influence the odour,
the flavour, the nutritional value and the resistance to
30 pathogens.

Numbered amongst these metabolites are the water soluble chemical compounds that are designated by the general term glucosinolates. Glucosinolates can be grouped into aliphatic glucosinolates (derived from the amino acid methionine),
5 indolyl glucosinolates (derived from isoleucine or threonine) and the aromatic glucosinolates (derived from phenylalanine).

The basic chemical structure of glucosinolates is depicted by the following chemical formula:



Formula 1: basic chemical structure of glucosinolates

15 wherein R is methionine, isoleucine, threonine or phenylalanine, which may be modified or elongated.

The route for synthesis of glucosinolates in plants has been revealed. The elongase enzyme, which is encoded by the gene BoGSL-ELONG, plays an important role in the synthesis of
20 glucosinolates. This enzyme catalyzes the stepped chain elongation of the glucosinolates. An example of the in vivo synthesis of aliphatic glucosinolates is described in figure 1.

Figure 1 shows that the amino acid methionine is
25 converted to homo-methionine. Various routes for synthesis are possible where the homo-methionine compound is taken as a starting point. Direct aldoxime formation, for example, leads to glucosinolates with a side chain of 3 carbon atoms. If elongase catalyzes one or two extra elongations of the
30 methionine prior to the aldoxime reaction, glucosinolates

with side chains of 4 or 5 carbon atoms, respectively, are produced. The enzymes involved in the synthesis of the glucosinolates are depicted in figure 1 as numbers which are specified below the figure.

5 By proceeding to use different amino acids in combination with various chain elongations and side chain modifying steps, it is possible to produce a large number of different glucosinolates, such as: glucoiberin (3-methylsulphinypropyl glucosinolate (3MSPG)), progoitrin,
10 sinigrin, glucoraphanin (4-methylsulphinylbutyl glucosinolate (4MSBG)), progoitrin, 4-hydroxybrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin.

When the plants of the Brassicaceae family are digested by humans and animals, the glucosinolates are broken down
15 hydrolytically in the gastro-intestinal tract by the myrosinase enzyme (which is secreted by the intestinal flora) into a multiplicity of compounds, such as nitriles, isothiocyanates, indoles, amines and thiocyanates, which are then absorbed by the body.

20 It is known that a number of these breakdown products, in particular the indoles, the isothiocyanates and the thiocyanates, have properties that are beneficial to health, and in particular anticarcinogenic properties. It is described in literature, for example, that isothiocyanates
25 induce the activity of phase II enzymes, which are known to be involved in the detoxification and secretion of harmful compounds. It is also known that isothiocyanates can induce a programmed cell death in carcinomas. There is also evidence in literature of a correlation between elevated
30 concentrations of indoles and thiocyanates in edible crops

and a reduced risk of developing intestinal cancer, amongst other things. Attention has been focused for some time on the level and type of glucosinolates in Brassica plants due to their beneficial effect on health.

5 It is known, in particular, that two glucosinolates, and specifically their breakdown products, have pronounced anticarcinogenic properties. These glucosinolates are generally referred to as glucoiberine (3-methylsulphinypropyl glucosinolate (3MSPG)) and
10 glucoraphanin (4-methylsulphinybutyl glucosinolate (4MSBG)).

 In the literature available to date there are descriptions of attempts to raise the levels of glucosinolates, and in particular glucoiberine (3-methylsulphinypropyl glucosinolate (3MSPG)) and
15 glucoraphanin (4-methylsulphinybutyl glucosinolate (4MSBG)).

 United States Patent US 6,340,784, for example, describes the use of the elevated level of glucoiberine (3-methylsulphinypropyl glucosinolate (3MSPG)) and glucoraphanin (4-methylsulphinybutyl glucosinolate (4MSBG))
20 observed in 'wild', non-cultivated members of the Brassica varieties Brassica villosa and B. drepanensis. In this patent specification an attempt is made to introduce these properties by means of crossings into the cultivated, 'edible' Brassica varieties.

25 However, using these non-cultivated 'wild' Brassica varieties has the following significant drawbacks:

- 1) The use of non-cultivated 'wild' varieties can also lead to the introduction of undesirable glucosinolates into the edible crops ultimately
30 produced. These may be, for example, glucosinolates

that determine flavour, glucosinolates with a potent anti-nutritive property, toxic glucosinolates, etc.

- 2) In addition to the introduction of undesirable glucosinolates through the non-cultivated 'wild' Brassica varieties into the cultivated 'edible' Brassica varieties, it is possible that other properties that are not associated with glucosinolates and that are usually unknown, such as toxins, increased susceptibility to pathogens, reduced fertility, lower yield of edible parts, etc. will also be introduced.
- 3) Due to the relatively large genetic distance of Brassica villosa and B. drepanensis from the cultivars, their use calls for a very long (often covering several years or even decennia) and therefore extremely costly programme of (back)crossings, selections and analyses for producing once again a cultivatable Brassica crop.
- 4) The non-cultivated 'wild' varieties often have morphological features which consumers find unattractive, such as a hairy leaf, an unappealing colour, deviating and unrecognisable edible plant parts, etc.

With regard to the introduction of undesirable properties of non-cultivated 'wild' Brassica varieties into cultivated 'edible' varieties, it is interesting to note the practice of Brassica napus breeding where the lowering of certain harmful glucosinolates is a specific goal with respect to the production of cultivated crops.

This applies in particular to crops that are intended as animal feed. If large quantities of such harmful glucosinolates are absorbed by animals, harmful side effects occur, for example in the thyroid gland. An accumulation of glucosinolates in the thyroid gland interferes with the synthesis of the thyroid hormone. Additionally, thiocyanates inhibit the absorption of iodine compounds by the thyroid gland.

It is therefore an object of the present invention to produce cultivated 'edible' plants belonging to the Brassicaceae family with elevated levels of anticarcinogenic glucosinolates without the aforementioned drawbacks.

This object is achieved according to the invention with the Brassica plants which are produced by the method as described in claim 1. Claim 1 describes a method comprising:

- a) providing a cultivated Brassica oleracea plant with elevated levels of anticarcinogenic glucosinolates in the edible parts of the Brassica oleracea plant;
- b) the use of the Brassica oleracea plant provided under a) as the starting material for breeding Brassica varieties with elevated levels of anticarcinogenic glucosinolates,

wherein the anticarcinogenic glucosinolates comprise at least glucoiberin (3-methylsulphanylpropyl glucosinolate (3MSPG)) and/or glucoraphanin (4-methylsulphanylbutyl glucosinolate (4MSBG)), and wherein the concentration of 3MSPG per 100 gram of fresh weight of the edible part is greater than 100 micromol and the concentration of 4MSBG per 100 gram of fresh weight of the edible part is greater than 50 micromol.

It is known of plants belonging to the cultivated Brassica oleracea group that they are safe for consumption ('safe use') by humans and animals. This means in actual practice that it is generally assumed that these plants do not contain any compounds that are harmful for either humans or animals. Since this group of plants has been used for many centuries, it is also known that they do not have any harmful side effects even when consumed over a long period of time (many decennia). As a consequence, the chance of introducing undesirable properties, such as other harmful or unwanted glucosinolates or toxins, is reduced to a minimum, and probably even to zero.

Many vegetables belonging to the Brassicaceae family belong to the Brassica oleracea group. Since the Brassica oleracea plant produced according to the present invention has a relatively small genetic distance, if any distance at all, from the cultivated 'edible' Brassica oleracea plants obtained with elevated levels of anticarcinogenic glucosinolates, the latter plants can be obtained simply according to step b) whereby genetic material is exchanged by means of crosses.

Nor does the use of a plant belonging to the Brassica oleracea group produce any 'edible' plants or parts of plants which consumers consider to be unpalatable.

There are many ways of providing a cultivated Brassica oleracea plant with elevated levels of anticarcinogenic glucosinolates in the edible parts.

For example, such a plant can be provided using molecular markers (hybridisation, restriction fragment length polymorphism (RFLP), PCR) and, in particular, by using

molecular markers which are associated with genes which encode for enzymes that are involved in the synthesis of glucosinolates with an anticarcinogenic effect. Such enzymes are known to the average skilled artisan since the route for synthesis of glucosinolates has been revealed (see also figure 1).

A further possibility for the provision of a Brassica oleracea plant according to the present invention is an analysis of the expression level of genes, and in particular of those genes which encode for enzymes that are involved in the synthesis of glucosinolates. A reduced or elevated expression of a specific gene can indicate an enhanced concentration of glucosinolates with an anticarcinogenic effect. There are many methods available in this field, such as real time PCR, Northern Blot analysis, quantitative PCR, etc., all of which are part of the practical skills and knowledge of the average skilled artisan.

It is also possible to provide a Brassica oleracea plant according to a) by means of a biochemical determination of the concentration of anticarcinogenic glucosinolates. An example of such a biochemical determination is High Performance Liquid Chromatography, or HPLC for short. The concentration and the nature of the glucosinolates present in a specific Brassica oleracea plant can be determined simply with the aid of a chromatogram which gives a graphical presentation of the detected data of the HPLC. Other examples of biochemical methods are colouration of specific glucosinolates, immunological methods which reveal specific glucosinolates in tissue samples, mass spectrometry, NMR, infrared absorption analysis, etc.

A Brassica oleracea plant according to the present invention can also be provided using modern molecular biological methods. Such methods can be used, for example, for the in vivo influence of the expression of genes which
5 encode for enzymes that are involved in the biosynthesis of glucosinolates. Examples of such methods are knock-out, knock-in, RNA silencing, anti-sense mRNA, etc.

Once a cultivated Brassica oleracea plant with elevated levels of anticarcinogenic glucosinolates has been provided,
10 it can be used to introduce this property into plants belonging to the Brassicaceae family. Possible breeding methods include cross-fertilizations, anther culture, micro trace culture, protoplast fusion and genetic modification, which are commonly known within the field so that the average
15 skilled artisan should have no difficulty choosing the most efficient method.

According to the present invention, the glucosinolates with an anticarcinogenic effect are the glucosinolates glucoiberine (3-methylsulphinypropyl glucosinolate (3MSPG))
20 and/or glucoraphanin (4-methylsulphinybutyl glucosinolate (4MSBG)). These glucosinolates have a very powerful anticarcinogenic effect. In order to increase the chance of producing a plant belonging to the Brassicaceae family with high levels of anticarcinogenic glucosinolates, the
25 concentration of glucoiberin (3-methylsulphinypropyl glucosinolate (3MSPG)) per 100 gram of fresh weight of the edible part should preferably be greater than 280 micromol, more preferably greater than 390 micromol and most preferably greater than 790 micromol. After all, there is always a
30 chance that during the aforementioned step b), part of the

high levels of anticarcinogenic glucosinolates that were originally produced will be lost.

This also applies with respect to glucoraphanin (methylsulphinylbutyl glucosinolate (4MSBG)). According to the present invention, the concentration of glucoraphanin (4-methylsulphinylbutyl glucosinolate (4MSBG)) per 100 gram of fresh weight of the edible part should preferably be greater than 120 micromol, and more preferably greater than 140 micromol.

The edible parts of the plant according to the invention include head cabbage (white, red and Savoy cabbage), stems (kohlrabi), cruciferous vegetables (broccoli, cauliflower and broccoli sprouts) and axillary buds (Brussels sprouts).

Several Brassica oleracea varieties are particularly suitable for use in the method according to the present invention. These are Savoy cabbage (Brassica oleracea convar. capitata var. sabauda), broccoli (Brassica oleracea convar. botrytis var. cymosa) and broccoli sprouts (Brassica oleracea convar. botrytis var. asparagoides). Of these varieties, broccoli sprouts are particularly suitable (Brassica oleracea convar. botrytis var. asparagoides).

Consumers prefer to eat fresh vegetables. Accordingly, broccoli sprouts enjoy favoured use since this variety exhibits very good cold hardiness as a consequence of which the variety can be grown throughout the year. This produces a constant supply (throughout the year) of fresh vegetables. Moreover, consumers prefer broccoli sprouts due to their familiar morphology.

The respective varieties of the races Wirosa (Savoy cabbage, annex 1), Belstar (broccoli, annex 2), Coronado

(broccoli, annex 3) and Bordeaux (broccoli sprouts, annex 4) are specifically preferred. These races are characterised according to the corresponding descriptions of these varieties according to article 11, para. 2 of the Vegetable Seed Directive of the European Community (70/458/EEC).

The method according to the present invention is particularly suitable for the provision of plants belonging to the Brassicaceae family, which plants are selected from the group comprising cauliflower or romanesco (Brassica oleracea convar. botrytis var. botrytis); broccoli (Brassica oleracea convar. botrytis var. cymosa); broccoli sprout (Brassica oleracea convar. botrytis var. asparagoides); Brussels sprouts (Brassica oleracea convar. oleracea var. gemmifera); white cabbage or oxheart cabbage (Brassica oleracea convar. capitata var. alba); red cabbage (Brassica oleracea convar. capitata var. rubra); Savoy cabbage (Brassica oleracea convar. capitata var. sabauda); kohlrabi (Brassica oleracea convar. acephala var. gongyloides); kale (Brassica oleracea convar. acephala var. sabellica); and Portuguese cabbage (Brassica oleracea var. trunchuda syn. costata).

The Brassica plants that are provided by the method according to the present invention have particularly desirable properties with respect to plants known from the art, and in particular having regard to their high levels of anticarcinogenic glucosinolates. The present invention therefore also relates to plants, seeds and parts of plants that can be obtained according to the method described above.

Due to their anticarcinogenic properties, the plants according to the present invention are particularly suitable for use in the preparation of a food product or

pharmaceutical composition that can be used for prophylaxis and/or treatment of cancer. Examples of such use are food products in the form of salads, juice, bars, meals, snacks, etc. For pharmaceutical compositions the plants could be incorporated into tablets, injectible liquids, suppositories, capsules, suspensions, carriers, sustained release carriers, etc.

The present invention will be explained further below by reference to a number of examples which are in no way intended to restrict the invention in any respect and which are only meant to illustrate possible embodiments of the present invention.

EXAMPLES

Example 1. starting material:

Plants of the various Brassica oleracea genotypes (see Table 1) all grew in the same field, hence in the same weather conditions and under an identical feeding regime. All plants received the same quantity of fertilizer (see Table 2). A total of 41 different Brassica oleracea genotypes were used.

TABLE 1. Brassica oleracea genotypes used

	Sowing date	Planting date	Sample taken
White cabbage			
Almanac	20 March	13 May	23 October
Krautman	20 March	13 May	23 October
Mentor	20 March	13 May	23 October

	Sowing date	Planting date	Sample taken
Mandy	20 March	13 May	23 October
Lennox	20 March	14 May	23 October
Danish 11-2	20 March	14 May	23 October
Red cabbage			
Integro	1 May	4 June	2 October
Azzuro	13 March	13 May	23 October
Huzaro	14 March	13 May	23 October
Buscaro	13 March	13 May	23 October
Pesaro	13 March	13 May	23 October
Oxheart cabbage			
Bejo 2574	14 June	15 July	2 October
Bejo 2575	28 June	29 July	2 October
Capricorn	14 June	15 July	2 October
Kohlrabi			
Kolibri	12 July	13 August	8 October
Korist	12 July	13 August	8 October
Broccoli			
Lucky	21 June	25 July	2 October
Alborada	21 June	25 July	2 October
Belstar	21 June	25 July	2 October
Surveyor	21 June	25 July	2 October
Coronado	21 June	25 July	8 October
Bordeaux	14 June	16 July	14 November
Cauliflower			
Jerez	7 June	9 July	2 October
Cassius	7 June	9 July	2 October
Encanto	7 June	9 July	2 October
Skywalker	31 May	4 July	2 October

	Sowing date	Planting date	Sample taken
Panther	7 June	8 July	2 October
Romanesco			
Bejo 1955	7 June	8 July	2 October
Veronica	7 June	8 July	2 October
Amfora	7 June	8 July	2 October
Kale			
Ripbor	17 May	13 June	23 October
Redbor	17 May	13 June	23 October
Brussels sprouts			
Franklin	1 March	24 April	8 October
Nautic	1 March	24 April	23 October
Maximus	1 March	24 April	23 October
Glenroy	1 March	24 April	23 October
Doric	1 March	24 April	23 October
Dominator	1 March	24 April	14 November
Revenge	1 March	24 April	14 November
Savoy cabbage			
Ovasa	3 May	5 June	2 October
Wirosa	3 May	5 June	2 October

TABLE 2. fertilization data

On 21 March 2002, a nitrogen sample was taken; the plot of land where the Brassica plants were planted has its own reserves of 70 kg of pure nitrogen.

Element	Pure fertilizer	Fertilizer type	Concentration
Magnesium	25 kg/ha	Kieserite	100 kg/ha

Element	Pure fertilizer	Fertilizer type	Concentration
Phosphate	300 kg/ha	Triple Super Phosphate	700 kg/ha
Potassium	300 kg/ha	Patent-Kali	1000 kg/ha
Nitrogen	200 kg/ha	Lime Saltpetre	500 kg/ha

Example 2. sampling

Five different plants or parts of a plant (leaf, sprouts, corolla) were harvested from each grown variety according to Table 1. Care was taken to avoid taking plants from the outer row in order to avoid peripheral effects.

Of the white cabbage varieties, 3 whole cabbages were harvested. Two facing segments, each being 1/8 of the cabbage, were taken from each cabbage. In the case of the broccoli varieties, 3 rosettes were cut from the centre and edge of the different plants. In the case of the sprouts, two facing quarters were taken as a sample from each plant.

The samples were then frozen using liquid nitrogen and pulverized. The resulting powder was stored at -20°C for further processing and analysis.

Example 3. Extraction of glucosinolates

5 grams of the powder obtained in example 2 was weighed and placed in 50ml centrifuge tubes, which were subsequently heated in a water bath to 75°C. Then 12ml of boiling methanol (100%) was added to the tubes and the suspension was mixed. 1.0 ml 3 mM of glucotropaeoline was then added immediately as an internal standard.

The sample was extracted in a water bath at 75°C for not less than 20 minutes and regularly shaken. Thereafter the

solid parts were pelleted by means of centrifugation (10 minutes, 5000x g) at room temperature and the supernatant was transferred to a clean centrifuge tube. The above extraction method was performed a further two times on the supernatant
5 obtained, each time with 10 ml of boiling methanol (70%) solution. The extract obtained was stored at -20°C.

Example 4. Desulpherization of glucosinolates

10 grams of DEAE Sephadex A-25 powder was measured out
10 and to it was added 80 ml 2M of acetic acid. The suspension was then stored overnight at room temperature without being stirred. The volume of the suspension was then doubled by the addition of 2 M of acetic acid. A 2 ml syringe was sealed at the bottom with a wad of glass wool. The DEAE Sephadex
15 suspension was carefully placed in this syringe until a column of approximately 1.5 ml had formed. The filled syringe was then transferred to a 10 ml test tube. Here the column was washed twice with 1 ml of water.

Approximately 2 ml of the supernatant was passed through
20 the column obtained, according to example 3. The column was then washed twice with 1 ml 20 mM NaAc solution (pH 4.0). The column was transferred to a clean tube and 75 :1 fresh sulphatase solution (25 mg Sulphatase type H-1 (Sigma s-9626) /ml bidest) was passed through the column. This enzyme was
25 allowed to act on the column for one night at room temperature. The desulphated glucosinolates were then eluated using 3 x 0.5 ml bidest and the combined fractions were filtered through a 0.45 :m filter (13mm, Alltech).

Example 5. HPLC analysis

For High Performance Liquid Chromatography (HPLC) analysis, use was made of equipment that permits gradient elution. A UV detector set to a wavelength of 229 nm was connected to this equipment. An Alltech Optiguard® 1 mm reversed phase C18 reversed phase column was used as a pre-column. A Novapak C18 column was used as a separating column.

The eluents used for the column were composed as follows:

1) Eluent A: 0.05% tetramethylammoniumchloride (Merck).

2) Eluent B: 0.05% tetramethylammoniumchloride in H₂O/Acetonitril (80/20 v/v).

The injection volume was 20 :l and the total flow rate was kept at a constant 1.0 ml/min. The gradient profile at which the eluents passed through the column was as follows:

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
1	100	0
21	0	100
26	100	0
31	100	0

After the eluent had passed through the column, the E₂₂₉ was measured with the aid of a UV detector.

Example 6. reference samples used

The following internal standards were used for internal reference purposes:

a) Glucotropaeolin (KLV, Denmark)

- b) Sinigrin
- c) Gluconasturtin
- d) Sprout (Cyrus)
- e) Rapeseed (Colza; BCR reference sample; No. 367R).

5

Example 7. determination of glucosinolate levels

The level of glucosinolates (GLS) was determined with respect to the internal standard (IS) and is expressed in micromol/100 gram fresh weight. The relative response factor (RRF) with respect to glucotropaeolin of the measured substances was determined. These data are summarised in Table 3. The concentration of glucosinolates in each sample was then determined with a correction being made using the relative response factor found. The results are presented in Table 4.

TABLE 3 Relative response factors with respect to glucotropaeolin

DESULPHOGLUCOSINOLATE	GLUCOTROPAEOLIN
glucoiberin	1.126
progoitrin	1.147
sinigrin	1.053
glucoalyssin	1.13
glucoraphanin	1.126
gluconapoleiferin	1.00
gluconapin	1.168
4-hydroxyglucobrassicin	0.295
glucotropaeolin	-

DESULPHOGLUCOSINOLATE	GLUCOTROPAEOLIN
glucobrassicin	0.526
glucosturtin	1.00
4-methoxyglucobrassicin	0.26
neoglucobrassicin	0.21

TABLE 4 Glucosinolate levels measured in tested Brassica oleracea genotypes.

- 5 All values were measured in duplicate and expressed as micromol glucosinolates per 100 gram of fresh weight. In literature, the level of glucosinolates is often represented as micromol/gram of dry weight. The values measured and the values found in literature can be resolved into one another
- 10 with the following conversion factor: cabbage has a dry material level of 7-15%; average 10%. Therefore, 100 gram of fresh weight corresponds (on average) to 10 gram of dry weight; accordingly, the values in the table must be divided by 10 in order to allow comparison with values in literature.

15

	Glucoiberin (3MSPG)	3MSPG (%)	Glucoraphanin (4MSBG)	4MSBG (%)	Other glucosinolates	Total glucosinolates
White cabbage						
Almanac	24.8	19.5%	18.9	14.9%	83.2	126.9
Krautman	67.7	39.2%	1.9	1.1%	102.9	172.5
Mentor	57.6	23.9%	3.1	1.3%	180.3	241.0
Mandy	100.0	42.9%	28.3	12.1%	105.0	233.3
Lennox	109.0	53.7%	18.8	9.3%	75.3	203.1
Danish 11-2	60.7	31.5%	3.8	2.0%	127.9	192.4

	Glucoiberin (3MSPG)	3MSPG (%)	Glucoraphanin (4MSBG)	4MSBG (%)	Other glucosinolates	Total glucosinolates
Red cabbage						
Integro	19.3	12.5%	33.9	22.0%	100.6	153.8
Azurro	13.8	14.8%	7.2	7.7%	72.0	93.0
Huzaro	13.1	7.7%	61.8	36.4%	94.9	169.8
Buscaro	36.4	15.1%	38.9	16.2%	165.0	240.3
Pesaro	31.2	14.6%	43.5	20.4%	138.9	213.6
Oxheart cabbage						
Bejo 2574	28.1	20.6%	0.7	0.5%	107.9	136.7
Bejo 2575	72.0	43.6%	9.2	5.6%	84.1	165.3
Capricorn	31.2	36.8%	5.0	5.9%	48.6	84.8
Kohlrabi						
Kolibri	12.8	23.3%	28.8%	52.4%	13.4	55.0
Korist	6.4	46.0%	0.0	0.0%	7.5	13.9
Broccoli						
Lucky	20.6	21.3%	35.8	37.0%	40.4	96.8
Alborada	25.8	18.1%	69.0	48.4%	47.7	142.5
Belstar	26.1	11.2%	129.7	55.5%	77.9	233.7
Surveyor	26.1	18.8%	57.8	41.7%	54.7	138.6
Coronado	52.1	19.7%	140.7	53.1%	72.1	264.9
Bordeaux	395.6	74.2%	26.7	5.0%	110.9	533.2
Cauliflower						
Jerez	16.8	36.5%	2.8	6.1%	26.4	46.0
Cassius	7.6	24.4%	0.7	2.3%	22.8	31.1
Encanto	10.5	25.2%	0.0	0.0%	31.1	41.6
Skywalker	10.2	31.4%	0.0	0.0%	22.3	32.5
Panther	34.2	57.8%	7.9	13.3%	17.1	59.2
Romanesco						
Bejo 1955	25.4	54.9%	2.1	4.5%	18.8	46.3
Veronica	15.9	32.9%	12.4	25.6%	20.1	48.4
Amfora	13	24.4%	16.0	30.1%	24.2	53.2
Kale						
Ripbor	35	35.0%	1.7	1.7%	63.4	100.1

	Glucoiberin (3MSPG)	3MSPG (%)	Glucoraphanin (4MSBG)	4MSBG (%)	Other glucosinolates	Total glucosinolates
Redbor	23.4	14.6%	0.0	0.0%	136.4	159.8
Brussels sprouts						
Franklin	51.1	9.9%	28.9	5.6%	437.3	517.3
Nautic	37	11.5%	41.8	13.0%	243.2	322.0
Maximus	91.5	31.9%	22.8	8.0%	172.2	286.5
Glenroy	43.6	12.9%	11.7	3.5%	283.1	338.4
Doric	38.2	6.7%	26.4	4.6%	504.1	568.7
Dominator	83.3	13.3%	9.2	1.5%	532.4	624.9
Revenge	47.5	9.9%	8.5	1.8%	422.9	478.9
Savoy cabbage						
Ovasa	55	54.3%	0.7	0.7%	45.8	101.5
Wirosa	284.8	59.7%	7.8	1.6%	183.8	476.4

Table 4 clearly shows high levels of glucoiberin (3-methylsulphanylpropyl glucosinolate (3MSPG)) in the Bordeaux (broccoli sprout), the Lennox (white cabbage), the Mandy
5 (white cabbage) and the Wirosa (Savoy cabbage), the values being 395.6 micromol, 284.8 micromol, 109.0 micromol and 100.0 micromol, respectively. There are high levels of glucoraphanin (4-methylsulphanylbutyl glucosinolate (4MSBG)) in the varieties Coronado (broccoli), Belstar (broccoli),
10 Alborada (broccoli) and Huzaro (red cabbage), the values being 140.7 micromol, 129.7 micromol, 69.0 micromol and 61.8 micromol, respectively.

Example 8

Data were gathered according to the same protocol at another location and another time. The results are presented in Table 5.

Genotype	Progoitrin	Sinigrin	4MSBG	3MSPG	Gluconapin
Brussels sprouts					
Maximus	27.3	67.6		154.9	7.6
Dominator	65.3	153.3		105.9	22.2
Broccoli					
Surveyor			47.1	18.1	
Bordeaux	2.7	72.1		796.8	
White cabbage					
Lennox	10.5	73.4		151.4	2.9

Annex I Wirosa

The Netherlands

FORM II

Ministry of Agriculture, Nature Management and Fisheries -

Bezuidenhoutseweg 73, The Hague

SUBJECT: Information according to article 11 par. 2 of the vegetable seed directive (70/458/EEC):
 ADMISSION OF A NEW VARIETY

- 10 1. Species: Brassica oleracea L. convar. capitata (L.) Alef. var. sabauda DC
 - Savoy cabbage
 2. Variety: Wirosa
 3. Maintainer: NL 8 - Bejo Zaden B.V.
 4. Date of admission:
 15 5. Indication of the variety: b
 6. Short description of the variety:

UPOV directive: TG/48/6

UPOV no.	Characteristic	Class	Code	Remarks
	Seedling: anthocyanic colouring hypocotyl	present	9	
1	Plant: height	low to medium	4	
2	Plant: maximum diameter (incl. wrapper leaf)	-	-	
25 3	Plant: outer stem length	short to medium	4	
4	Plant: wrapper leaf attitude	half-raised	5	
5	Wrapper leaf: size	-	-	
6	Wrapper leaf: blade shape	round	3	to reverse egg-shaped
30 7	Wrapper leaf: profile upper side of blade	cupping	1	weak
8	Wrapper leaf: knobbling	medium to pronounced	6	fine
9	Wrapper leaf: knob size	small	3	
10	Wrapper leaf: folding	-	-	
11	Wrapper leaf: colour (with waxy layer)	grey-green	3	
35 12	Wrapper leaf: colour intensity	dark	7	
14	Wrapper leaf: waxy layer	strong	7	
15	Wrapper leaf: leaf margin undulation	weak	3	
16	Wrapper leaf: leaf margin notching**	-	-	
17	Wrapper leaf : leaf margin crimping**	-	-	
40	Cabbage: size	small to medium	4	
18 G	Cabbage: shape of longitudinal section	flattened circular	2	to circular
19	Cabbage: shape of base	-	-	
20	Cabbage: length	short	3	to medium-long
21	Cabbage: diameter	small to medium	4	
45 22	Cabbage: location of largest diameter	above centre	1	to centre
23	Cabbage: closure	half-closed	2	
24	Cabbage: bract knobbling	medium	5	

	UPOV no.	Characteristic	Class	Code	Remarks
	25	Cabbage: bract crimping	-	-	
	26	Cabbage: colour of bract	green	2	
5	27	Cabbage: colour intensity of bract	light to medium	4	
	28	Cabbage: bract anthocyanin content	weak	3	
	29	Cabbage: inner colour	-	-	
	31	Cabbage: firmness	firm	7	
	32	Cabbage: internal structure	-	-	
10	33	Cabbage: inner stem length	long	7	
	34 G	Harvest maturity	late	7	
	35	Cracks in cabbage after harvest time	-	-	
	36	Fusarium oxysporum f. sp. conglutinans fysio	-	-	
15	Distinctiveness:				
			Most similar to Hiversa, but with a shorter stem, a flatter leaf attitude and earlier formation of cabbage.		
20	7. Denomination in trials:		Wirosa		

Annex II Bordeaux

The Netherlands

FORM II

Ministry of Agriculture, Nature Management and Fisheries -

Bezuidenhoutseweg 73, The Hague

SUBJECT: Information according to article 11 par. 2 of the vegetable seed directive (70/458/EEC):
ADMISSION OF A NEW VARIETY

- 10 1. Species: Brassica oleracea L. convar. botrytis (L.) Alef. var. cymosa Duch
- Broccoli
2. Variety: Belstar
3. Maintainer: NL 8 - H Bejo Zaden B.V.
4. Date of admission: 17-08-2000
- 15 5. Indication of the variety: b
6. Short description of the variety:

UPOV directive: TG/151/3

20	UPOV no.	Characteristic	Class	Code	Remarks
	1	Plant: number of stems	one	1	
	2	Plant: height	medium	5	
	3	Leaf: attitude	half-raised	3	
25	4	Leaf: length	medium	5	
	5	Leaf: width	medium	5	
		Leaf: shape	elliptical	5	
	6	Leaf: number of lobes	few	3	
	7	Leaf blade: colour	grey-green	2	
30	8	Leaf blade: colour intensity	medium	5	
		Leaf: waxy layer	medium	5	
	9	Leaf blade: anthocyanic colouring	absent	1	
	10	Leaf blade: margin undulation	weak	3	
	11	Leaf blade: margin indentation	very shallow to shallow	2	
35	12	Leaf blade: knobbling	weak	3	
		Leaf blade: knob size	medium to large	6	
	13	Leafstalk: anthocyanic colouring	absent	1	
	14	Leafstalk: length	medium	5	
	15	Flower head: length of bifurcations at base	short	3	
40	16	Flower head: size	medium	5	
	17	Flower head: shape	circular	1	to flattened circular
	18 G	Flower head: colour	grey-green	3	
	19	Flower head: colour intensity	medium	5	
45	20	Flower head: anthocyanic colouring	absent	1	
	21	Flower head: intensity of anthocyanic colouring	-	-	

	UPOV no.	Characteristic	Class	Code	Remarks
	22	Flower head: scragginess	fine to medium	4	
	23	Flower head: granularity	fine to medium	4	
5	24	Flower head: firmness	firm	7	
	25	Flower head: bracteate	absent	1	
	26	Plant: secondary flower heads	present	9	
	27	Plant: presence of secondary flower heads	very weak to weak	2	
	28	Flower: colour	yellow	2	
10	29	Flower: intensity of yellow colour	medium to dark	6	
	30	Harvest maturity	late	7	to medium
	31	Start of flowering	medium to late	6	
		Type	annual	1	
15	Distinctiveness:		No comparable races. The race is characterised by a slightly lobed leaf, a medium grey-green flower head with fine to medium granularity and a rather late harvest maturity.		
20	7. Denomination in trials:		Bejo 1848		

Annex III Belstar

The Netherlands

FORM II

Ministry of Agriculture, Nature Management and Fisheries -

5

Bezuidenhoutseweg 73, The Hague

SUBJECT: Information according to article 11 par. 2 of the vegetable seed directive (70/458/EEC):
 ADMISSION OF A NEW VARIETY

- 10 1. Species: Brassica oleracea L. convar. botrytis (L.) Alef. var. cymosa Duch.
 - Broccoli
 2. Variety: Coronado
 3. Maintainer: NL 8 - H. Bejo Zaden B.V.
 4. Date of admission: 30/04/1997
 15 5. Indication of the variety: b
 6. Short description of the variety:

UPOV directive: TG/151/3

20	UPOV no.	Characteristic	Class	Code	Remarks
	1	Plant: number of stems	one	1	
	2	Plant: height	medium	5	
	3	Leaf: attitude	half-raised	3	
25	4	Leaf: length	medium	5	
	5	Leaf: width	medium to broad	6	
		Leaf: shape	elliptical to broad elliptical	6	
	6	Leaf: number of lobes	medium	5	
30	7	Leaf blade: colour	grey-green	2	
	8	Leaf blade: colour intensity	Medium to dark	6	
		Leaf: waxy layer	strong	7	
		Leaf blade: anthocyanic colouring	absent	1	
	10	Leaf blade: margin undulation	very weak to weak	2	
35	11	Leaf blade: margin indentation	shallow	3	
	12	Leaf blade: knobbling	very weak to weak	2	
		Leaf blade: knob size	medium	5	
	13	Leafstalk: anthocyanic colouring	absent	1	
	14	Leafstalk: length	short to medium	4	
40	15	Flower head: length of bifurcations at base	short	3	
	16	Flower head: size	medium	5	
	17	Flower head: shape	circular	1	
	18 G	Flower head: colour	grey-green	3	
	19	Flower head: colour intensity	medium to dark	6	
45	20	Flower head: anthocyanic colouring	absent	1	
	21	Flower head: intensity of anthocyanic colouring	-	-	

UPOV no.	Characteristic	Class	Code	Remarks
22	Flower head: scragginess	medium	5	
23	Flower head: granularity	fine to medium	4	
24	Flower head: firmness	medium	5	
25	Flower head: bracteate	absent	1	
26	Plant: secondary flower heads	absent	1	
27	Plant: presence of secondary flower heads	-	-	
28	Flower: colour	yellow	2	
29	Flower: intensity of yellow colour	light to medium	4	
30	Harvest maturity	very late	9	to late
31	Start of flowering	-	-	
	Fusarium oxysporum f. sp. conglutinans f. sp. resistant		9	

Distinctiveness:

No comparable races. The race is characterised by a half-raised leaf with very slight knobbling and a pronounced waxy layer, a circular, grey-green, medium firm flower head and a very late to late harvest maturity.

7. Denomination in trials:

Bejo 1744

Annex IV Coronado

The Netherlands

FORM II

Ministry of Agriculture, Nature Management and Fisheries -

5

Bezuidenhoutseweg 73, The Hague

SUBJECT: Information according to article 11 par. 2 of the vegetable seed directive (70/458/EEC):
 ADMISSION OF A NEW VARIETY

- 10 1. Species: Brassica oleracea L. convar. botrytis (L.) Alef. var. cymosa Duch
 - Broccoli
2. Variety: Bordeaux
3. Maintainer: NL 8c - H. Bejo Zaden B.V./Elso
- 15 4. Date of admission: 17/08/2000
5. Indication of the variety: b
6. Short description of the variety:

UPOV directive: TG/151/3

20	UPOV no.	Characteristic	Class	Code	Remarks
	1	Plant: number of stems	one	1	
	2	Plant: height	very high	9	
	3	Leaf: attitude	raised to half-		
25			raised	2	
	4	Leaf: length	medium	5	
	5	Leaf: width	narrow	3	
		Leaf: shape	narrow elliptical	3	
	6	Leaf: number of lobes	very many	9	
30	7	Leaf blade: colour	blue-green	3	
	8	Leaf blade: colour intensity	very dark	9	
		Leaf: waxy layer	very strong	9	
	9	Leaf blade: anthocyanic colouring	absent	1	
	10	Leaf blade: margin undulation	medium	5	
35	11	Leaf blade: margin indentation	shallow	3	
	12	Leaf blade: knobbling	very weak to weak	2	
		Leaf blade: knob size	very small to small	2	
	13	Leafstalk: anthocyanic colouring	present	9	
	14	Leafstalk: length	short	3	
40	15	Flower head: length of bifurcations at base	very long	9	
	16	Flower head: size	very small	1	
	17	Flower head: shape	-	-	
	18 G	Flower head: colour	violet	5	
	19	Flower head: colour intensity	medium	5	
45	20	Flower head: anthocyanic colouring	present	9	
	21	Flower head: intensity of anthocyanic colouring	very strong	9	

	UPOV no.	Characteristic	Class	Code	Remarks
	22	Flower head: scragginess	-	-	
	23	Flower head: granularity	-	-	
5	24	Flower head: firmness	very floppy	1	
	25	Flower head: bracteate	-	-	
	26	Plant: secondary flower heads	present	9	
	27	Plant: presence of secondary flower heads	very strong	9	
	28	Flower: colour	yellow	2	
10	29	Flower: intensity of yellow colour	-	-	
	30	Harvest maturity	very late	9	
	31	Start of flowering	very late	9	
		Type	annual	1	
15	Distinctiveness:				
	No comparable races. The race is characterised by a very tall plant, a very dark blue-green, narrow leaf with very many lobes, and very small purple flower heads (type purple sprouting broccoli).				
20	7. Denomination in trials:				
	BE 1891				
25					

CLAIMS

1. A method for the providing of a plant belonging to the Brassica family with elevated levels of anticarcinogenic glucosinolates, comprising:

a) providing a cultivated Brassica oleracea plant with elevated levels of anticarcinogenic glucosinolates in the edible parts of the Brassica oleracea plant;

a) the use of the Brassica oleracea plant provided under a) as the starting material for the breeding of Brassica varieties with elevated levels of anticarcinogenic glucosinolates, wherein the anticarcinogenic glucosinolates comprise at least glucoiberin (3-methylsulphinypropyl glucosinolate (3MSPG)) and/or glucoraphanin (4-methylsulphinybutyl glucosinolate (4MSBG)), and wherein the concentration of 3MSPG per 100 gram of fresh weight of the edible part is greater than 100 micromol and the concentration of 4MSBG per 100 gram of fresh weight of the edible part is greater than 50 micromol.

2. The method according to claim 1, wherein the concentration of 3MSPG per 100 gram of fresh weight of the edible part is greater than 280 micromol.

3. The method according to claim 1 or claim 2, wherein the concentration of 3MSPG per 100 gram of fresh weight of the edible part is greater than 390 micromol.
- 5
4. The method according to claim 1, claim 2 or claim 3, wherein the concentration of 3MSPG per 100 gram of fresh weight of the edible part is greater than 790 micromol.
- 10
5. The method according to claim 1, wherein the concentration of 4MSBG per 100 gram of fresh weight of the edible part is greater than 120 micromol.
- 15
6. The method according to claim 5, wherein the concentration of 4MSBG per 100 gram of fresh weight of the edible part is greater than 140 micromol.
- 20
7. The method according to any of the claims 1-6, wherein the cultivated Brassica oleracea plant is broccoli sprout (Brassica oleracea convar. botrytis var. asparagoides).
- 25
8. The method according to claim 7, wherein the broccoli sprout (Brassica oleracea convar. botrytis var. asparagoides) variety is Bordeaux.
9. The method according to any of the claims 1-6, wherein the cultivated Brassica oleracea plant is

Savoy cabbage (Brassica oleracea convar. capitata var. sabauda).

- 5 10. The method according to claim 9, wherein the Savoy cabbage (Brassica oleracea convar. capitata var. sabauda) variety is Wirosa.
- 10 11. The method according to any of the claims 1-6, wherein the cultivated Brassica oleracea plant is broccoli (Brassica oleracea convar. botrytis var. cymosa).
- 15 12. The method according to claim 11, wherein the broccoli (Brassica oleracea convar. botrytis var. cymosa) variety is Belstar or Coronado.
- 20 13. The method according to claims 1-12, wherein the plant belonging to the Brassica family is selected from the group consisting of cauliflower or romanesco (Brassica oleracea convar. botrytis var. botrytis); broccoli (Brassica oleracea convar. botrytis var. cymosa); broccoli sprout (Brassica oleracea convar. botrytis var. asparagoides); Brussels sprouts (Brassica oleracea convar. oleracea var. gemmifera);
- 25 white cabbage or oxheart cabbage (Brassica oleracea convar. capitata var. alba); red cabbage (Brassica oleracea convar. capitata var. rubra); Savoy cabbage (Brassica oleracea convar. capitata var. sabauda); kohlrabi (Brassica oleracea convar. acephala var. gongyloides); kale (Brassica oleracea convar.
- 30

acephala var. sabellica); and Portuguese cabbage
(Brassica oleracea var. trunchuda syn. costata).

- 5 14. A plant belonging to the Brassica family obtainable
by the method of any of claims 1-13.
15. Seeds of a plant belonging to the Brassica family
obtainable by the method of any of claims 1-13.
- 10 16. Parts of a plant belonging to the Brassica family
obtainable the method of any of claims 1-13.
- 15 17. The use of the plant, seeds or parts of a plant
according to one of claims 14-16 for the preparation
of a food product and/or pharmaceutical composition
for prophylaxis and/or treatment of cancer.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01H1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99/52345 A (PLANT BIOSCIENCE LTD) 21 October 1999 (1999-10-21) cited in the application the whole document	1-17
Y	BRANCA FERDINANDO ET AL: "Survey of aliphatic glucosinolates in Sicilian wild and cultivated Brassicaceae" April 2002 (2002-04), PHYTOCHEMISTRY (OXFORD), VOL. 59, NR. 7, PAGE(S) 717-724 , XP002289628 ISSN: 0031-9422 the whole document	1-17
Y	WO 03/004619 A (QUIROS CARLOS ;LI GENYI (US); UNIV CALIFORNIA (US)) 16 January 2003 (2003-01-16) pages 8,10,11,45 Examples, Tables, ----- -/-	1-17

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"P" document published prior to the International filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the International search

23 July 2004

Date of mailing of the International search report

06/08/2004

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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